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Effects of Specimen Collection Methodologies and Storage Conditions on the Short-Term Stability of Oral Microbiome Taxonomy

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ABSTRACT

Community profiling of the oral microbiome requires the recovery of quality sequences in order to accurately describe microbial community structure and composition. Our objective was to assess the effects of specimen collection method, storage medium, and storage conditions on the relative abundance of taxa in saliva and plaque identified using 16S rRNA genes. We also assessed short-term changes in taxon composition and relative abundance and compared the salivary and dental plaque communities in children and adults. Over a 2-week period, four successive saliva and dental plaque specimens were collected from four adults with no dental decay (108 samples), and two successive specimens were collected from six children with four or more erupted teeth (48 samples). There were minimal differences in community composition at the phylum and operational taxonomic unit levels between dental plaque collection using a scaler and collection using a CytoSoft brush. Plaque samples stored in OMNIgene medium showed higher within-sample Shannon diversity, were compositionally different, and were more similar to each other than plaque stored in liquid dental transport medium. Saliva samples stored in OMNIgene recovered similar communities for at least a week following storage at room temperature. However, the microbial communities recovered from plaque and saliva stored in OMNIgene were significantly different in composition from their counterparts stored in liquid dental transport medium. Dental plaque communities collected from the same tooth type over four successive visits from the same adult did not significantly differ in structure or composition.

IMPORTANCE

Large-scale epidemiologic studies require collection over time and space, often with multiple teams collecting, storing, and processing data. Therefore, it is essential to understand how sensitive study results are to modest changes in collection and storage protocols that may occur with variation in personnel, resources available at a study site, and shipping requirements. The research presented in this paper measures the effects of multiple storage parameters and collection methodologies on the measured ecology of the oral microbiome from healthy adults and children. These results will potentially enable investigators to conduct oral microbiome studies at maximal efficiency by guiding informed administrative decisions pertaining to the necessary field or clinical work.

The human oral microbiome includes the microbial communities that live on the tooth enamel, tongue, cheek, palate, tonsils, and gums; each of these niches hosts compositionally diverse microorganisms: viruses, fungi, archaea, bacteria, and protozoa (1–3), in a variety of community structures. Perturbations of oral microbiota are associated with many common dental conditions, including periodontal disease (4), gingivitis (5, 6), and dental caries (7). A number of studies show statistical associations between poor oral health and coronary heart disease (8), endocarditis, preterm birth (9), and exacerbations of existing chronic conditions, such as diabetes and osteoporosis (10), although whether these associations are causal is uncertain.

A comprehensive understanding of the interactions of oral microbiota with environmental and host factors may help disentangle the role the oral microbiome plays in health and disease (2). To do so requires large-scale epidemiologic studies, which require collection over time and space, often with multiple teams collecting, storing, and processing data. Therefore, it is essential to understand how sensitive study results are to modest changes in collection and storage protocols that may occur with variation in personnel, resources available at a study site, and shipping requirements. Understanding the effects of collection method, storage medium, and storage conditions is essential to properly plan and conduct these studies.

Sample storage, holding temperatures, and collection methods may differentially affect bacterial DNA recovery and, consequently, the interpretation of ecological outcomes from downstream sequencing (11), potentially biasing conclusions. Previous studies have evaluated the effect of storage conditions on 16S metagenomic analysis on fecal specimens (12, 13) and vaginal swabs (14). For example, a study of 11 fecal samples suggested that short-term storage conditions affected DNA integrity and resulting microbial compositions (12). In contrast, a comparison of fecal samples from two patients with irritable bowel syndrome and two

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TABLE 1 Sample collection schedule[a]

Visit	Sample type	Participant	Medium	Method	Site(s) sampled (tooth no.)	Storage before processing
1st	Plaque	Adult	LDTM	Scaler	Molars (17, 26)	−80°C for 2 days and then −20°C
1st	Plaque	Adult	LDTM	Scaler	Premolars (34, 45)	−80°C for 2 days and then −20°C
1st	Plaque	Adult	OMNIgene	Scaler	Molars (36, 47)	Room temp
1st	Plaque	Adult	OMNIgene	Scaler	Premolars (15, 24)	Room temp
1st	Plaque	Adult	LDTM	CytoSoft	Molars (16, 27)	−80°C for 2 days and then −20°C
1st	Plaque	Adult	LDTM	CytoSoft	Premolars (35, 44)	−80°C for 2 days and then −20°C
1st	Plaque	Adult	OMNIgene	CytoSoft	Molars (37, 46)	Room temp
1st	Plaque	Adult	OMNIgene	CytoSoft	Premolars (14, 25)	Room temp
2nd	Plaque	Adult	LDTM	Scaler	Molars (16, 27)	−80°C for 2 days and then −20°C
2nd	Plaque	Adult	LDTM	Scaler	Premolars (35, 44)	−80°C for 2 days and then −20°C
2nd	Plaque	Adult	OMNIgene	Scaler	Molars (37, 46)	Room temp
2nd	Plaque	Adult	OMNIgene	Scaler	Premolars (14, 25)	Room temp
2nd	Plaque	Adult	LDTM	CytoSoft	Molars (17, 26)	−80°C for 2 days and then −20°C
2nd	Plaque	Adult	LDTM	CytoSoft	Premolars (34, 45)	−80°C for 2 days and then −20°C
2nd	Plaque	Adult	OMNIgene	CytoSoft	Molars (36, 47)	Room temp
2nd	Plaque	Adult	OMNIgene	CytoSoft	Premolars (15, 24)	Room temp
3rd	Plaque	Adult	LDTM	Scaler	Pooled molars (26, 27, 36, 37)	−80°C for 2 days and then −20°C
3rd	Plaque	Adult	LDTM	CytoSoft	Pooled molars (16, 17, 46, 47)	−80°C for 2 days and then −20°C
4th	Plaque	Adult	OMNIgene	Scaler	Pooled molars (26, 27, 36, 37)	Room temp
4th	Plaque	Adult	OMNIgene	CytoSoft	Pooled molars (16, 17, 46, 47)	Room temp
1st	Saliva	Adult	OMNIgene	Stimulated	Saliva	−20°C
1st	Saliva	Adult	OMNIgene	Stimulated	Saliva	RT for 2 days and then −20°C
1st	Saliva	Adult	OMNIgene	Stimulated	Saliva	RT for 5 days and then −20°C
1st	Saliva	Adult	OMNIgene	Stimulated	Saliva	RT 7 days and then −20°C
1st	Saliva	Adult	LDTM	Stimulated	Saliva	−20°C
1st	Saliva	Adult	LDTM	Stimulated	Saliva	RT for 2 days and then −20°C
1st	Saliva	Adult	LDTM	Stimulated	Saliva	−80°C for 2 days and then −20°C
1st	Gum	Child	OMNIgene	Swab	Gingival gum	−20°C
1st	Saliva	Child	OMNIgene	Swab	Saliva	−20°C
1st	Plaque	Child	OMNIgene	CytoSoft	Any erupted tooth	−20°C
1st	Plaque	Child	OMNIgene	CytoSoft	3 other teeth	−20°C
2nd	Gum	Child	OMNIgene	Swab	Gingival gum	Room temp
2nd	Saliva	Child	OMNIgene	Swab	Saliva	Room temp
2nd	Plaque	Child	OMNIgene	CytoSoft	Any erupted tooth	Room temp
2nd	Plaque	Child	OMNIgene	CytoSoft	3 other teeth	Room temp

[a] Schedule of sample collection from 4 healthy adults and 6 children by visit, sample type, collection methodology, and storage before processing. There was a total 27 samples collected from each adult and 8 from each children, for a total of 156 samples over 4 visits. Indicated on the map in Fig. S3 in the supplemental material are the teeth from which plaque samples were collected.

healthy controls showed that whether stored at room temperature for 24 h or at −80°C for 6 months, the microbial communities clustered with their host of origin (13). Similarly, a study of vaginal swabs from 8 women found no significant differences in vaginal bacterial community compositions among samples stored under different conditions (14).

To the best of our knowledge, none of the previously published studies profiling the bacterial community of plaque and saliva (specimens widely used for oral microbiome studies) have evaluated the effects of collection methods, storage medium, and storage conditions on salivary and plaque bacterial communities.

To assess the variability associated with different collection and storage conditions, we conducted a study designed to answer the three following technical questions.

(i) Does collection using dental scalers rather than cytology brushes (CytoSoft; Medical Packaging Corporation) modify the structure and composition of bacterial communities recovered from plaque?

(ii) Does storage medium (liquid dental transport medium [LDTM] or the commercially available OMNIgene collection kit) modify the structure and composition of the plaque and saliva bacterial communities recovered?

(iii) Does storage temperature modify the structure and composition of the saliva bacterial communities recovered?

Last, our study design gave us an opportunity to comment on short-term changes in the adult plaque microbiome and the congruence between bacterial communities from plaque and saliva samples among healthy adults and healthy children.

MATERIALS AND METHODS

Participant selection and sampling. We recruited a convenience sample (nonprobability easily available sample) from 4 adults (age 19 to 29 years) and 6 children (age 1 to 4 years). Half of the adults and half of the children were female. Adults were chosen from individuals with 2 or fewer restorations and no active lesions; children had one or more teeth. Each adult was seen for 4 visits over a maximum of 2 weeks and each child for 2 visits over a maximum of 2 weeks. The schedule of sample collection from adults and children, by visit, is detailed in Table 1. Because there were multiple visits over the 2-week period, and we were concerned that sampling might result in short-term changes in the plaque community, the collection of adult plaque included alternate molar and premolar group-

ings on successive visits. Plaque was collected from occlusal, lingual, or buccal surfaces using either a cytology brush or a dental scaler. Adult plaque from visits 3 and 4 was pooled from the same teeth sampled in visits 1 and 2 to ensure that there were sufficient quantities of DNA and that a single tooth would not influence the results. For children, the schedule included sampling a single tooth, as we wished to determine if the community from a single child tooth could be characterized.

Plaque from an individual was stored separately, except where indicated. All adult plaque placed in an OMNIgene 501 kit was stored at room temperature, in accordance with the manufacturer's recommendation. Adult plaque placed into Anaerobe Systems PRAS liquid dental transport medium (LDTM) AS-916 was stored at −80°C for 2 days before being moved to −20°C until DNA extraction. Saliva was collected on an adult's first visit and aliquoted into 7 samples to be stored in either LDTM or OMNIgene under various temperatures and durations prior to DNA extraction, as shown in Table 1. All child samples were stored in OMNIgene. In total, 156 samples were collected, but 151 samples were included in the final pool due to 5 child samples with inadequate DNA extracted.

Ethics, consent, and permissions. The University of Pittsburgh institutional review board approved the study protocol; informed consent was obtained from participating adults and a parental guardian of each participating child.

Sample DNA extraction and measuring DNA concentration. DNA was extracted using the DNeasy blood and tissue kit in concert with the QIAcube, with both the kit and equipment manufactured by Qiagen (Venlo, The Netherlands). The QIAcube is an automated benchtop apparatus that runs a number of Qiagen protocols that reduce the variability introduced by manual extraction. One hundred microliters of each sample was mixed with enzyme cocktail and was incubated at 37°C for 60 min, with intermittent vortexing every 10 min. The enzyme cocktail is composed of Promega (Madison, WI, USA) cell lysis solution, lysozyme, mutanolysin, RNase A, and lysostaphin in 22.5:4.5:1.125:1.125:1 parts, respectively. After recovery, DNA was measured using a NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at −80°C. We reextracted all samples with a post-PCR DNA concentration of less than 5 ng/μl, averaged from two NanoDrop 2000C measurements.

Amplifying and barcoding the V6 region of the 16S rRNA gene. There is no clear consensus in the literature regarding which variable region of the 16S rRNA region is best for oral microbiome studies. Our choice of V6 on the Illumina sequencing platform enabled high coverage of the entire region. We used a previously published primer set to amplify the V6 region of the bacterial 16S rRNA gene that maximizes overlap in paired-end reads generated from an Illumina sequencing platform (15). The forward primer is 5′-TCCWACGCGARGAACCTTAACC-3′, and the reverse primer is 5′-CAACRACACGAGCTGACGAC-3′. In order to sequence >100 samples in multiplex, the reverse primer was modified by adding barcodes. These 8-nucleotide error-correcting barcodes were designed to optimize amplification, with consideration given to G+C content, self-complementation, and elimination of homopolymers of 6 or more nucleotides (16). Thus, each of our reverse primers is a unique identifier of the sample it is used to amplify, with a barcoded signature at the beginning of the 5′ region: 5′-BBBBBBBBCAACRACACGAGCTGACGAC-3′, where BBBBBBBB is the barcode.

Following the barcoding assignment, 25 ng of DNA was used as the template to amplify the V6 region. We used 25 μl of Promega's GoTaq green, 2.5 μl of 10 μM forward primer, and 2.5 μl of 10 μM reverse primer, filled to a final reaction volume of 50 μl with PCR-grade water for each sample. The PCR mixture was amplified using an S1000 thermocycler (Bio-Rad, Hercules, CA, USA), with a modified protocol that added 10 cycles with a decreasing gradient of annealing temperature starting at 61°C down to 51°C. After these 10 cycles, 24 cycles at 94°C for 45 s, 5°C for 45 s, and 72°C for 45 s were applied, for a total of 35 cycles. The denaturing and extension temperatures as well as the denaturing and extension times were the same for each cycle. After amplification, 6 μl of the PCR product was loaded onto 1% agarose gels and run at 100 V to visualize the presence of a band around the 130-bp region. Successfully amplified PCR products were purified, requantified using the NanoDrop 2000C, and stored at −20°C. Any sample averaging less than 5 ng/μl from two measurements was reamplified. If low DNA concentrations persisted, DNA was reextracted from the sample.

Sequencing the V6 region. Three nanograms of barcoded DNA from each visually confirmed, purified, and requantified sample was pooled into a single 1.5-ml Eppendorf tube. A barcoded mock community was also added to the pool for quality control of sequencing. The mock community consisted of 57 species, with an overrepresentation of genera typically and atypically found in abundance in a healthy human mouth (see Table S1 in the supplemental material). The distribution of observed and expected phylum and genus percentages of the mock community is shown in Table S2 in the supplemental material. Since the reference database is specialized for the oral microbiome, sequences with the mock community barcode were extracted and operational taxonomic units (OTU) repicked with Greengenes 13.8 (17). The pool was concentrated to 55 ng/μl using Amicon centrifugal filter units for benchtop centrifuges (Millipore, Billerica, MA, USA). As a reliability check, the pool was split into 2 equal volumes. The two technical replicates were sequenced on separate lanes of an Illumina HiSeq platform with 100 paired-end cycles at the University of Michigan Sequencing Core, Ann Arbor, MI.

Upstream processing: from raw reads to OTU table. The raw sequences were processed and analyzed on the university-wide Flux high-performance-computing cluster. The resources available to us were 9 processing cores with 25 Gb of physical memory allocated to each. The paired ends were joined using FLASH (18), which is designed to join paired ends of small fragments less than twice the length of a read. After joining the paired ends, the sequences were demultiplexed and filtered using QIIME 1.9.0 (19). We removed reads with more than 135 nucleotides (our expected read length was between 110 and 130 nucleotides, including barcodes, adaptors, and primers), with missing quality scores, where the number of ambiguous bases exceeded 6, where the mean quality score was below 25 across the entire read, where the number of mismatches in the forward or reverse primers exceeded 0, or where barcodes with ambiguous nucleotides could not be error corrected based on our population of barcodes.

We used as a reference database, CORE, a specialized database for the identification of bacteria within the oral microbiome (20). Singleton OTU were filtered out as part of the default QIIME parameters. In addition, we excluded all OTU with <0.05% relative abundance. After the OTU table was generated, taxonomy was assigned using the RDP Classifier (21) in QIIME. The sequences were then aligned with the CORE-aligned sequences as the template using PyNAST (22). Sequences that failed to align were omitted from the subsequent tree and OTU table construction.

As a reliability check, we sequenced two technical replicates. The first replicate generated 71,968,207 pairs of raw sequences at 101 bp. After joining the paired ends, 70,505,638 sequences (124 bp ± 5 bp) remained. The second technical replicate generated 136,396,554 pairs of raw sequences, 131,807,313 of which remained after joining the paired ends. Both runs were quality filtered and demultiplexed to verify that the distributions of read counts for each sample were roughly the same in the two runs. After the replicates were determined to be nearly identical, the raw joined sequences from the two sequence runs were combined and demultiplexed. A total of 171,758,509 reads were successfully demultiplexed into a unique sample and passed all of our quality-filtering criteria. This translates to roughly 85% recovery of quality joined paired-end reads from the raw sequence file. These numbers are consistent with a study analyzing error and biases associated with Illumina HiSeq platforms, which showed that removal of ~12.5% of the data via quality filtering reduced error rates 7-fold (23). Here, we employed stringent quality-filtering parameters and discarded ~15% of our alignable sequences. After filtering OTU that constituted <0.05% relative abundance, there remained 168,616,157 reads representing 749 OTU. The number of reads mapped to a sample ranged from 240 to 9,533,688, with 147/152 samples mapped to more than 10,000

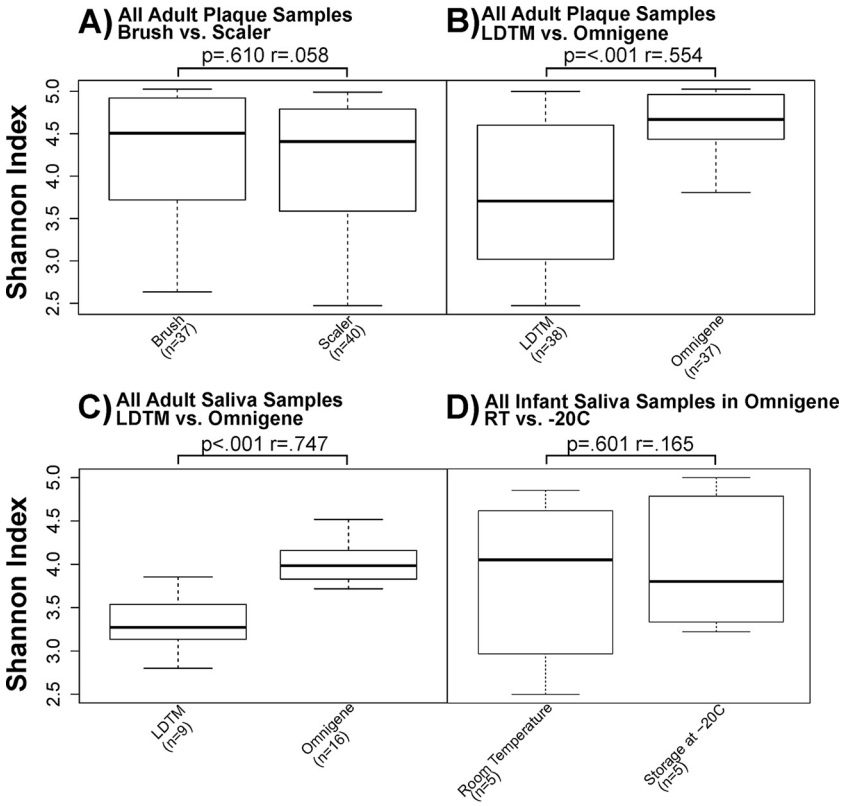

FIG 1 Shannon diversity indices of plaque and saliva. Oral specimens collected from 4 healthy adults and 6 children. (A) The diversity in plaque collected using a CytoSoft brush was similar to the diversity in plaque collected using a scaler. (B and C) Microbial communities isolated from plaque or saliva stored in OMNIgene rather than liquid dental transport medium (LDTM) were more diverse. (D) Diversities of child saliva microbial communities stored in OMNIgene at room temperature (RT) and at −20°C were similar. Analysis includes multiple samples from the same individual (see Table 3). Each box shows the 25th to 75th percentiles of the distribution, with the central line indicating the median. The whiskers show the minimum and maximum values, excluding outliers. The boundaries for outliers were determined by adding the product of 1.5 and the interquartile range to quartile 3 and subtracting it from quartile 1. Outliers are denoted by singular points. Statistical differences were tested using Kruskal-Wallis testing with a P value of <0.05, and intergroup effect sizes (r) are indicated.

reads. The wide distribution of reads to a multiplexed sample is not uncommon and is seen in other studies using Illumina platforms (24, 25). Samples where more than 50% of the reads could not be assigned a bacterial phylum were deemed to be artifacts of sequencing and subsequently excluded from analysis. There were two such samples, giving a final study size of 149 oral specimens and one mock community.

Downstream analysis. For downstream analysis and graphic generation, we used the Phyloseq 1.10.0 R package (26) and QIIME 1.9.0 scripts (27). Taxonomic summaries, diversity, and distance metrics were derived from the OTU table constructed by QIIME in the upstream analytical processing. The derivative OTU table, taxonomy, phylogeny, and reference sequences used were outputs from QIIME's pick_open_reference_otus.py script. The same mapping file of sample barcode and metadata used in the demultiplexing step was also used in further downstream analyses. We normalized the varied numbers of sequences obtained per sample by using compositional approaches. There were 5 samples (4 adult and 1 child) that did not surpass 10,000 reads/sample and were excluded from further analyses.

Alpha and beta diversity metrics and testing. Alpha diversity is a measure of diversity within samples or within groups. We used Shannon's index to estimate the diversity within a sample. This metric takes into account the relative abundance of OTU rather than simple presence. The Kruskal-Wallis nonparametric test was performed on each of the panels to test for community differences in the mean number of observed OTU by groupings of interest. Significance threshold was set at an α value of 0.05.

Beta diversity is a measure of diversity between samples or between groups. We used Euclidean distance and weighted UniFrac distance to assess the diversity between samples and groups of samples. Within-group and between-group average Euclidean distances were compared to each other and tested with the Kruskal-Wallis test adjusted for multiple comparisons. The significance threshold for this test was set at an α value of 0.05. A nonparametric Adonis test was performed on the weighted UniFrac distance matrix to determine whether the sample group tested was deterministic of the variation seen in the distances.

Linear mixed-effects regression. We created separate linear mixed-effects models to predict the $\log_{10}$-transformed relative abundances of the 8 most common phyla among adult plaque samples with medium, method, and site of plaque collection as fixed effects and participant and visit as random effects. Likewise, we created separate linear mixed-effects models to predict the $\log_{10}$-transformed relative abundance of the 8 most common phyla for child specimens, with oral site and storage temperature as fixed effects and participant and visit as random effects. Child samples included biological but no technical replicates.

Differential abundance testing. To test for differential abundance in a nonnormalized OTU table, we used the nonparametric Wald negative binomial test available in the differential_abundance.py script in QIIME 1.9.0 or the DESeq2 package in R. A diagnostic dispersion estimate plot was created for each comparison to ensure the validity of the statistical test.

RESULTS

Study participants were healthy adults and children with minimal active lesions and fillings. All sampled surfaces were sound, as determined by trained dental hygienists.

TABLE 2 Differences in community structure

Site/storage conditions by sample group	Comparison group 1 (no. of specimens)	Comparison group 2 (no. of specimens)	R^2	Adonis test P value[a]
Adult sites				
	Adult molar plaque (32)	Adult pooled plaque (16)	0.004	0.941
	Adult molar plaque (32)	Adult premolar plaque (29)	0.015	0.585
	Adult molar plaque (32)	Adult saliva (25)	0.220	<0.001
	Adult pooled plaque (16)	Adult premolar plaque (29)	0.041	0.281
	Adult pooled plaque (16)	Adult saliva (25)	0.282	<0.001
	Adult premolar plaque (29)	Adult saliva (25)	0.247	<0.001
Child sites				
	Child gum (11)	Child saliva (10)	0.167	0.004
	Child gum (11)	Plaque (1 tooth) (11)	0.130	0.017
	Child gum (11)	Plaque (4 teeth) (10)	0.150	0.014
	Child saliva (10)	Plaque (1 tooth) (11)	0.248	<0.001
	Child saliva (10)	Plaque (4 teeth) (10)	0.229	<0.001
	Plaque (1 tooth) (11)	Plaque (4 teeth) (10)	0.055	0.332
Child storage conditions				
	Gum at −20°C (5)	Gum at RT (6)	0.147	0.107
	Child saliva at −20°C (5)	Child saliva at RT (5)	0.210	0.063
	Plaque (1 tooth) at −20°C (5)	Plaque (1 tooth) at RT (6)	0.194	0.135
	Plaque (4 teeth) at −20°C (5)	Plaque (4 teeth) at RT (5)	0.401	<0.001
Adult plaque				
	OMNIgene (39)	LDTM (38)	0.257	<0.001
	Scaler (40)	CytoSoft (37)	0.007	0.627
Adult saliva				
	OMNIgene (16)	LDTM (9)	0.249	<0.001
Adult saliva in LDTM				
	−20°C (3)	RT for 2 days (3)	0.108	0.800
	−20°C (3)	Dry ice (3)	0.040	0.900
	RT for 2 days (3)	Dry ice (3)	0.031	0.900
Adult saliva in OMNIgene				
	−20°C (4)	RT for 2 days (4)	0.061	0.841
	−20°C (4)	RT for 5 days (4)	0.099	0.570
	−20°C (4)	RT for 7 days (4)	0.116	0.453
	RT for 2 days (4)	RT for 5 days (4)	0.033	1.000
	RT for 2 days (4)	RT for 7 days (4)	0.076	0.749
	RT for 5 days (4)	RT for 7 days (4)	0.049	0.976

[a] Adonis testing using weighted UniFrac to determine differences in community composition by adult sites, child sites, child sample storage conditions, adult sample storage conditions, and adult saliva storage temperatures.

Does collection using a dental scaler rather than CytoSoft brushes modify the structure and composition of bacterial communities recovered from plaque? The average ± standard deviation (SD) DNA concentrations following extraction, as measured by NanoDrop 2000C, were not significantly different for the scaler-collected and CytoSoft brush-collected plaque samples (4.5 ± 2.6 ng/μl versus 4.8 ± 2.5 ng/μl; $P > 0.05$), nor were the average ± SD DNA concentrations following PCR concentration (8.4 ± 2.8 ng/μl for scalar versus 8.7 ± 3.2 ng/μl for CytoSoft brush; $P > 0.05$). Due to low DNA concentration values, extraction was redone for 13 (32.5%) of the dental scaler samples and 18 (29.5%) of the CytoSoft brush samples. Three CytoSoft brush-recovered plaque specimens were excluded from analysis because the total number of reads mapping to those specimens was less than our resampling depth of 10,000 reads.

The microbial diversities of adult plaque, as measured using Shannon's index, were similar for specimens collected using the dental scaler (4.2, $n = 40$) and CytoSoft brush (4.2, $n = 37$) (Fig. 1A; Kruskal-Wallis, $P = 0.62$). There were no detectable differences in community structure between the two methods of plaque recovery (Table 2; see also Fig. S1A in the supplemental material; Adonis test, $P = 0.63$). However, the mean Euclidean distances between all adult plaque collected by a CytoSoft brush were lower than the Euclidean distances between all adult plaque samples collected by dental scalers (Fig. 2A; Kruskal-Wallis, $P = 0.03$), suggesting slightly higher precision in community recovery when using a CytoSoft brush.

The two recovery methods also did not differ in relative abundance of bacterial phyla (Fig. 3A). After controlling for visit, participant, oral site, collection method, and storage media, there was no statistically significant difference in the relative abundances of the 8 most common phyla when using a dental scaler compared to

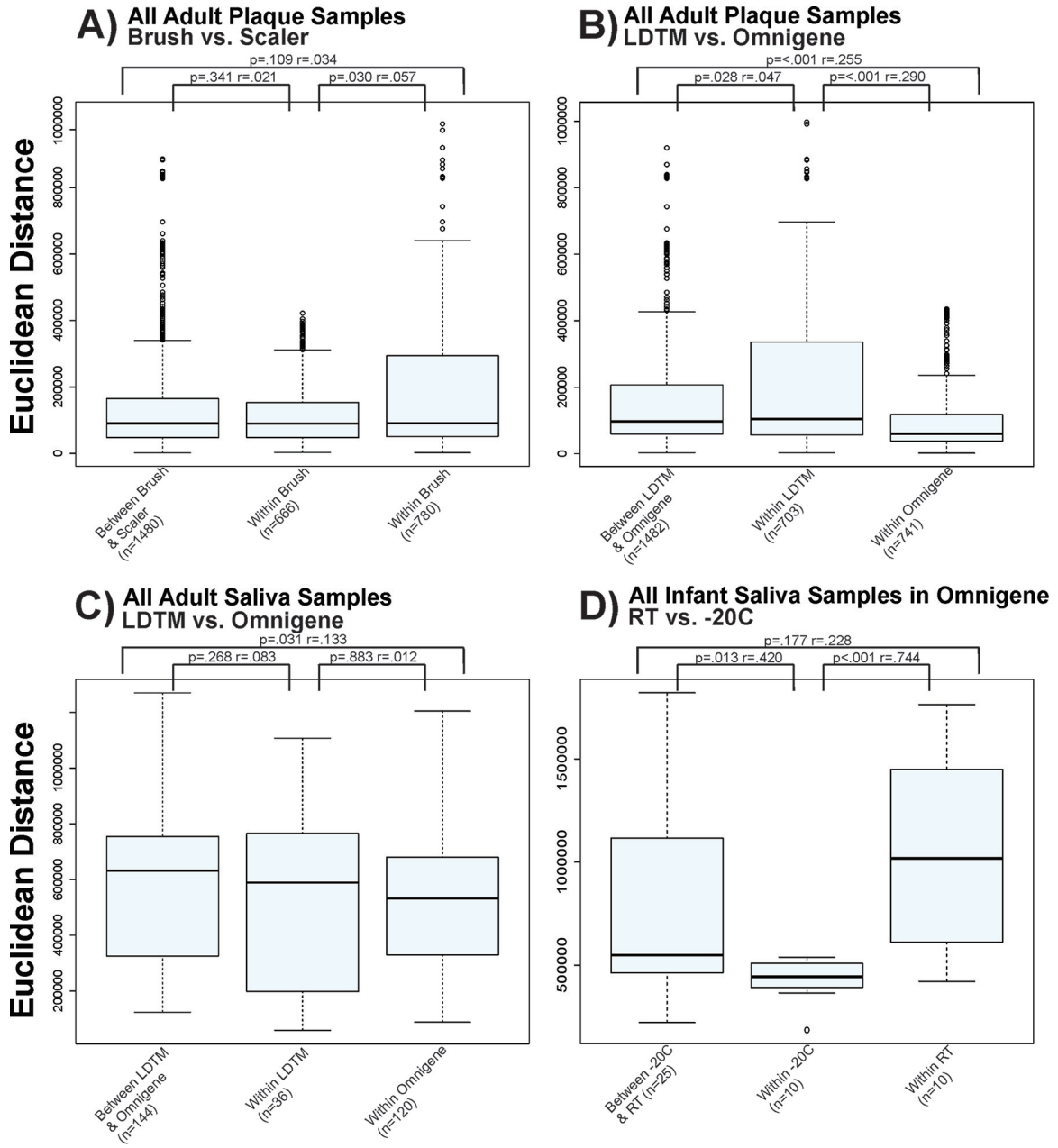

FIG 2 Euclidean distances within and between samples. Comparisons by plaque collection method (A), plaque storage medium (B), saliva storage medium (C), and storage temperature (D) of child saliva samples in OMNIgene. Oral specimens were collected from 4 healthy adults and 6 children. The number of samples compared is shown below each box plot. Analysis includes multiple samples from the same individual (see Table 3). Each box shows the 25th to 75th percentiles of the distribution, with the central line indicating the median. The whiskers show the minimum and maximum values, excluding outliers. The boundaries for outliers were determined by adding the product of 1.5 and the interquartile range to quartile 3 and subtracting it from quartile 1. Outliers are denoted by singular points. Statistical differences were tested using Kruskal-Wallis testing with a P value of <0.05, and intergroup effect sizes (r) are indicated.

the CytoSoft brush (Table 3). With respect to relative abundance at the OTU level, 17 out of 749 OTU were differentially abundant between the two recovery methods (Table 4).

Does storage in LDTM or the OMNIgene collection kit modify the structure and composition of plaque and saliva bacterial communities recovered? Similar amounts of DNA were recovered from both adult plaque and saliva samples stored in OMNIgene and LDTM (average ± SD DNA concentrations, 6.1 ± 5.6 ng/μl and 5.9 ± 2.9 ng/μl, respectively; $P > 0.05$). Extractions were redone for 27 (27.3%) of the samples stored in OMNIgene and 7 (25%) of the samples stored in LDTM. The average ± SD DNA concentrations post-PCR amplification were significantly higher for samples stored in OMNIgene than in LDTM (9.7 ± 3.9 ng/μl versus 8.7 ± 2.8 ng/μl, respectively; $P = 0.05$). Further, the plaque and saliva microbial communities recovered among adult samples stored in OMNIgene storage medium were significantly more diverse than those stored in LDTM ($P < 0.001$; Fig. 1B and C).

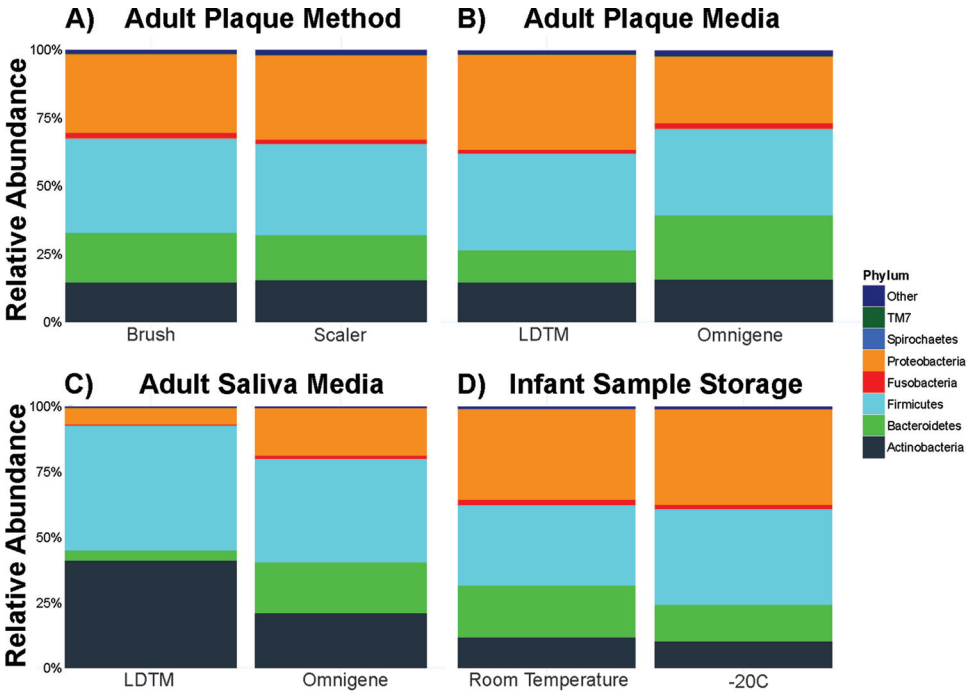

FIG 3 Relative phylum abundance of oral samples. Oral specimens collected from 4 healthy adults and 6 children. (A) We observed no difference in microbial communities recovered from plaque collected by a scaler or CytoSoft. (B and C) There were observable differences between communities isolated from LDTM and OMNIgene. (D) There were also observable differences among child saliva and plaque stored in OMNIgene and held at room temperature compared to −20°C. Analysis includes multiple samples from the same individual (see Table 3).

Adult plaque and saliva microbial communities clustered by storage medium (nonmetric multidimensional scaling [NMDS] plots; see Fig. S1 in the supplemental material). Further, the Euclidean distances of adult plaque samples stored in OMNIgene were significantly lower than those of plaque samples stored in LDTM ($P < 0.001$; Fig. 2B). The distributions of phylum relative abundances between the two storage media are visually distinct for the top 4 phyla in the plaque and saliva samples (Fig. 3B and C). The most notable difference for adult plaque samples was that the relative abundance of *Bacteroidetes* recovered from OMNIgene was nearly twice that of *Bacteroidetes* recovered from LDTM, and the relative abundances of *Proteobacteria* and *Firmicutes* of plaque stored in OMNIgene were lower than those recovered from LDTM. This was also observed in the mixed models adjusting for site, method, medium, and random effects, where the $\log_{10}$ relative abundances of the 8 most common phyla were significantly different for plaque and for many phyla for saliva when stored in OMNIgene compared to LDTM (Table 3). At the OTU level, 167 OTU were differentially abundant between adult plaque samples stored in OMNIgene and adult plaque samples stored in LDTM (Table 4). For adult saliva technical replicates, 316 OTU were differentially abundant between OMNIgene and LDTM storage. Overall, for both adult plaque and saliva, the choice of medium affected the composition of the recovered community (Table 2).

Does storage temperature modify the structure and composition of bacterial communities recovered? Child saliva samples stored in OMNIgene at room temperature were not significantly more diverse than those held at −20°C (Fig. 1D), but the two temperatures affected the relative abundance of phyla (Fig. 3D). However, the mean Euclidean distances were less variable for samples stored at −20°C than at room temperature ($P < 0.001$; Fig. 2D and Table 2). In the linear mixed-effects regression models controlling for participant random effects and oral site (plaque and saliva versus gum), storage at −20°C was a significant predictor of recovering a lower relative abundance of *Bacteroidetes* and TM7 candidate division organisms, whereas storage at −20°C predicted a higher, but insignificant, relative abundance of *Firmicutes* and *Proteobacteria* (Table 3). The effects of storage conditions on community composition were most apparent for pooled plaque (Table 2). Bacterial diversity within adult saliva stored in OMNIgene was not affected by storage temperature (see Fig. S4 in the supplemental material).

We observed no difference in the diversity of the 4 adult saliva samples that were stored at −20°C until shipment or at room temperature for 2, 5, and 7 days before shipment. We also observed no difference in diversity for 4 adult samples of saliva stored in LDTM at −20°C, on dry ice, and at room temperature for 2 days before shipment. For adult saliva stored in OMNIgene at room temperature, the community recovered remained stable for up to a week. Storage temperature also did not affect the community recovered from adult saliva in LDTM (Table 2). However, storage at room temperature for 5 to 7 days compared to that at −20°C significantly reduced the relative abundance of *Firmicutes*, and storage on dry ice rather than −20°C reduced the relative abundance of unclassified (other) phyla (Table 3).

Are bacterial communities from adult and child plaque samples reflected in their saliva? Saliva bacterial communities could be clearly distinguished from the plaque samples in adults and children (Table 2). In adults, the number of differentially abun-

TABLE 3 Linear mixed-effects models predicting phylum relative abundance[a]

Patient group and specimen type(s) (no. of specimens)	Phylum relative abundance or mixed-effects model parameter	Replicate category or collection method	Value for:							
			Firmicutes	*Proteobacteria*	*Actinobacteria*	*Bacteroidetes*	*Fusobacteria*	Other	TM7	*Spirochaetes*
Adult plaque (80)	Relative abundance (%)		38.3	21.5	21.4	16.4	1.3	0.85	0.15	0.03
	Random-effect variance	Visit	0.000	0.003	0.008	0.002	0.019	0.026	0.000	0.025
		Participant	0.001	0.000	0.000	0.017	0.049	0.004	0.032	0.076
		Residual	0.011	0.014	0.064	0.127	0.212	0.058	0.195	0.233
	Fixed-effect estimate (*P* value)	Scaler (vs CytoSoft)	0.018 (0.433)	−<0.001 (0.974)	−0.083 (0.181)	0.023 (0.826)	−0.052 (0.624)	−0.007 (0.903)	−0.003 (0.957)	−0.078 (0.500)
		OMNIgene (vs LDTM)	−0.086 (<0.001)	−0.117 (<0.001)	0.227 (<0.001)	0.451 (<0.001)	0.256 (0.020)	0.328 (<0.001)	0.449 (<0.001)	0.602 (<0.001)
		Pooled plaque from 4 molars (vs 1 molar)	0.020 (0.515)	−0.043 (0.318)	−0.028 (0.722)	0.051 (0.719)	0.100 (0.493)	0.102 (0.269)	0.172 (0.198)	0.002 (0.971)
		Plaque from 1 premolar (vs 1 molar)	−0.014 (0.582)	0.001 (0.961)	0.113 (0.110)	−0.024 (0.844)	−0.014 (0.890)	−0.045 (0.574)	−0.149 (0.174)	−0.192 (0.147)
Adult saliva (26)	Relative abundance (%)		41.6	25.9	15.7	15.3	1.0	0.4	0.09	1.13E−4
	Random-effect variance	Participant	0.015	0.003	0.000	0.005	0.000	0.009	0.145	0.039
		Residual	0.007	0.046	0.090	0.070	0.111	0.080	0.103	0.203
	Fixed-effect estimate (*P* value)	OMNIgene (vs LDTM)	−0.107 (0.016)	−0.150 (0.175)	0.974 (<0.001)	0.411 (0.003)	0.712 (<0.001)	−0.250 (0.087)	−0.206 (0.215)	0.450 (0.054)
		Storage at RT for 2 days (vs storage at −20°C)	−0.063 (0.155)	0.190 (0.088)	0.084 (0.578)	−0.054 (0.680)	0.050 (0.756)	−0.223 (0.125)	0.152 (0.358)	−0.234 (0.313)
		Storage at RT for 5 days (vs storage at −20°C)	−0.166 (0.004)	0.212 (0.135)	0.058 (0.755)	0.069 (0.678)	0.102 (0.634)	−0.078 (0.663)	−0.041 (0.833)	−0.256 (0.385)
		Storage at RT for 7 days (vs storage at −20°C)	−0.108 (0.056)	0.083 (0.552)	0.156 (0.425)	0.092 (0.589)	0.127 (0.556)	−0.100 (0.583)	0.285 (0.179)	−0.032 (0.898)
		Storage in dry ice (vs storage at −20°C)	−0.038 (0.529)	0.055 (0.709)	0.141 (0.505)	0.040 (0.818)	0.084 (0.713)	−0.448 (0.029)	0.445 (0.059)	0.090 (0.770)
Child plaque, saliva, and gum (43)	Relative abundance (%)		35.278	32.499	18.098	11.348	2.071	0.567	0.116	0.023
	Random-effect variance	Participant	0.000	0.001	0.000	0.000	0.021	0.000	0.007	0.000
		Residual	0.020	0.022	0.071	0.099	0.129	0.133	0.324	0.374
	Fixed-effect estimate (*P* value)	Plaque from 1 tooth (vs child gum)	0.009 (0.860)	0.049 (0.433)	−0.235 (0.038)	0.027 (0.821)	−0.189 (0.211)	0.174 (0.258)	0.073 (0.746)	0.206 (0.419)
		Plaque from 4 teeth (vs child gum)	0.093 (0.131)	−0.088 (0.177)	−0.247 (0.033)	0.110 (0.417)	−0.043 (0.771)	−0.280 (0.077)	0.048 (0.830)	−0.046 (0.847)
		Child saliva (vs child gum)	0.060 (0.314)	0.047 (0.451)	−0.184 (0.103)	0.057 (0.661)	−0.035 (0.802)	−0.501 (0.001)	−0.472 (0.051)	−0.654 (−0.012)
		Storage at −20°C	0.049 (0.260)	0.062 (0.172)	−0.271 (<0.001)	−0.145 (0.129)	−0.208 (0.060)	−0.080 (0.462)	−0.449 (0.010)	−0.260 (0.162)

[a] Linear mixed-effects regression models predicting $\log_{10}$ transformation of relative abundance of each of the most common phyla, taking into account replicates (participant), collection method, and plaque type.

TABLE 4 Numbers of differentially abundant OTU (adjusted P value < 0.05)[a]

Site/storage conditions by sample group	Comparison group 1 (no. of specimens)	Comparison group 2 (no. of specimens)	No. of different OTU								
			Total	*Actinobacteria*	*Bacteroidetes*	*Firmicutes*	*Fusobacteria*	*Proteobacteria*	*Spirochaetes*	TM7	Other phyla
Adult sites											
	Adult molar plaque (32)	Adult pooled plaque (16)	13	8	0	3	0	2	0	0	0
	Adult molar plaque (32)	Adult premolar plaque (29)	10	2	1	2	0	4	0	1	0
	Adult molar plaque (32)	Adult saliva (25)	551	76	108	210	24	106	4	3	20
	Adult pooled plaque (16)	Adult premolar plaque (29)	8	7	1	0	0	0	0	0	0
	Adult pooled plaque (16)	Adult saliva (25)	537	87	107	192	26	103	1	3	18
	Adult premolar plaque (29)	Adult saliva (25)	521	83	100	192	24	102	2	1	17
Child sites											
	Child gum (11)	Child saliva (10)	96	4	7	38	3	33	0	0	11
	Child gum (11)	Plaque (1 tooth) (11)	133	17	31	44	10	30	0	0	1
	Child gum (11)	Plaque (4 teeth) (10)	127	28	20	40	9	29	0	0	1
	Child saliva (10)	Plaque (1 tooth) (11)	318	30	48	129	10	79	3	0	19
	Child saliva (10)	Plaque (4 teeth) (10)	325	61	58	119	10	61	4	5	7
	Plaque (1 tooth) (11)	Plaque (4 teeth) (10)	47	14	13	7	1	12	0	0	0
Child storage conditions											
	Gum at −20°C (5)	Gum at RT (6)	1	1	0	0	0	0	0	0	0
	Child saliva at −20°C (5)	Child saliva at RT (5)	9	0	0	6	2	0	0	0	1
	Plaque (1 tooth) at −20°C (5)	Plaque (1 tooth) at RT (6)	99	9	17	28	7	34	0	0	4
	Plaque (4 teeth) at −20°C (5)	Plaque (4 teeth) at RT (5)	53	2	5	21	0	23	0	0	2
Adult plaque											
	OMNIgene (39)	LDTM (38)	167	16	36	47	8	48	0	1	11
	Scaler (40)	Cytology brush (37)	17	5	1	3	1	5	0	0	2
Adult saliva											
	OMNIgene (16)	LDTM (9)	316	38	56	127	8	72	0	2	13
Adult saliva in LDTM											
	−20°C (3)	RT for 2 days (3)	43	1	0	23	0	17	0	1	1
	−20°C (3)	Dry ice (3)	0	0	0	0	0	0	0	0	0
	RT for 2 days (3)	Dry ice (3)	0	0	0	0	0	0	0	0	0
Adult saliva in OMNIgene											
	−20°C (4)	RT for 2 days (4)	0	0	0	0	0	0	0	0	0
	−20°C (4)	RT for 5 days (4)	0	0	0	0	0	0	0	0	0
	−20°C (4)	RT for 7 days (4)	0	0	0	0	0	0	0	0	0
	RT for 2 days (4)	RT for 5 days (4)	0	0	0	0	0	0	0	0	0
	RT for 2 days (4)	RT for 7 days (4)	0	0	0	0	0	0	0	0	0
	RT for 5 days (4)	RT for 7 days (4)	0	0	0	0	0	0	0	0	0

[a] Wald negative binomial test detecting differentially abundant OTU by adult sites, child sites, child sample storage conditions, adult sample storage conditions, and adult saliva storage temperatures. The total number of OTU tested between each comparison group is 749.

dant OTU between saliva and plaque ranged from 521 to 551 total OTU. In contrast, the number of differently abundant OTU found in a comparison of different plaque sites in adults was 8 to 13 (Table 4). Child saliva compared to plaque (1 tooth) and pooled plaque (4 teeth) yielded 318 and 325 differentially abundant OTU, respectively. Child plaque samples from 1 tooth compared to pooled plaque (4 teeth) had 47 differentially abundant OTU.

How stable are saliva and plaque communities over short periods? In the mixed models predicting the relative abundance of the 8 most common phyla, there was minimal effect of visit for adult plaque (Table 3). This suggests that these communities are stable over short periods. Further, although samples were collected from different (but symmetrical) molars and premolars at different visits, we could not distinguish these communities from each other.

DISCUSSION

The effects of storage conditions and collection method have been evaluated for studies of the fecal and vaginal microbiome (12–14), but we found no studies assessing the effects of storage conditions and collection method on the bacterial microbiome in saliva and dental plaque. Our study fills that gap, evaluating the effects of plaque collection methodologies, oral specimen storage medium, and holding temperature in two distinct age groups, adults and children.

Our conclusions are as follows. First, the microbial communities collected using CytoSoft brushes were essentially the same as those collected using a dental scaler. Second, plaque and saliva bacterial communities recovered from samples stored using the OMNIgene 501 kit compared to LDTM were more diverse. Third, saliva specimens stored in OMNIgene could be held at room temperature for at least a week without differential recovery of microbial communities, although the relative abundances of some phyla were affected. We also found no differences in community recovery from saliva stored in LDTM at different temperatures. Bacterial communities from child plaque and saliva specimens stored in OMNIgene at room temperature were more diverse than those held at −20°C, but this was not true for adult saliva samples. Child samples may have lower bacterial loads and be more subject to sampling error when they were split for processing. Nevertheless, regardless of method and storage conditions, the most common phyla observed in children and adults were similar to those reported by other oral microbiome studies using healthy participants and stimulated saliva and plaque: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* (28, 29).

Our design also allowed us to demonstrate that the salivary and plaque microbiomes were very stable during the 2 weeks of this study. We sampled the 4 participating adults 4 times over a 2-week period, and the 6 participating children were sampled twice over a 2-week period. Visit had no effect on the relative abundances of phyla. Visit also had no effect on the average Euclidean distances between adult plaque samples, suggesting microbial community stability over the 4 visits of adult plaque collection spanning 2 weeks (see Fig. S5 in the supplemental material). These results are consistent with previous studies that used different collection, initial processing, and storage protocols but also found the salivary and plaque microbiome to be stable over short periods. Belstrøm et al. found that the salivary microbial profiles in 5 individuals were stable over 24 h and after 1 week (30). Similarly, Cameron et al. showed that the individual variation of the salivary microbiome from 10 participants sampled 7 times over 1 year was minimal compared to the variation between participants (31). Supragingival plaque also is stable for months (32). We compared plaque and salivary microbial communities and found, consistent with previous studies, that plaque communities contained more bacterial species (28, 33).

Our study was designed to optimize protocols for large-scale epidemiologic studies where samples have to be transported before processing. Although one might argue that we should have included a sample that was flash-frozen as a comparison, per the manufacturer's recommendation, OMNIgene is not amenable to flash freezing. We did not design this experiment for a comparison to one using no medium, as our primary purpose was to assess the effects of storage conditions on the two media. This is a limitation of this study. However, whatever medium is selected should be used for all samples, as recovery did vary by medium even when stored optimally. The differences between communities recovered using a dental scaler and CytoSoft were minimal but might be observable across a large sample, so using a single method is recommended. We used the same sequencing and postrun analytic methods for all samples to ensure comparability between groups, so we cannot evaluate the effects of these procedures on the study results. However, previous work suggests that the stringency of read quality filtering and clustering thresholds can substantially modify the observed microbial community structure (34). Our study design also allowed us to assess differences by the order in which teeth were sampled, and between molars and premolars. Although the number of adult participants was small, the number of samples collected per individual was sufficient to determine that these factors have minimal impact. This is consistent with other similar studies conducted using different microbiomes (35–37).

In summary, consistent with results of studies of the fecal and vaginal microbiome, we observed minimal effects on microbial composition and structure when samples were held at different temperatures (13, 14).

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We declare no conflicts of interest.

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